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Tan, Ee Yau, Arifullah, Mohamad and Soon, Jan Mei ORCID: 0000-0003-0488-1434 (2016) Identification of Escherichia coli strains from water vending machines of Kelantan, Malaysia using 16S rRNA gene sequence analysis. Exposure and Health . ISSN 1876-1658

Identification of *Escherichia coli* strains from water vending machines of Kelantan, Malaysia using 16S rRNA gene sequence analysis

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Abstract

Water vending machines provide an alternative source of clean and safe drinking water to the consumers. However, the quality of drinking water may alter due to contamination from lack of hygienic practices and maintenance of the machines. Hence this study was conducted to determine the microbiological quality of water from vending machines and associated contact surfaces. Seventeen water samples and 85 swab samples (nozzles, drip trays, coin slots, buttons and doors) from 3 locations in Kelantan were collected. Polymerase Chain Reaction (PCR) amplification and 16S ribosomal ribonucleic acid (rRNA) sequencing were carried out and sequences obtained were compared against the sequences available in the National Centre for Biotechnology Information (NCBI) database using the Basic Local Alignment Search Tool (BLAST) program. Coliform counts were observed in 94.12% of water samples, 76.47% of nozzles and 82.35% of drip tray swabs. Furthermore, results of 16S rRNA sequence analysis indicated that two gram-negative isolates were identified as *Escherichia coli* U 5/41 (Accession no. NR_024570.1) and *Escherichia coli* O157:H7 EDL933 (Accession no. CP008957.1) with similarity value of 100% respectively. The results from this study further improve our understanding of the potential microorganisms in drinking water. Regular maintenance and cleaning of water vending machines are important to reduce bacterial growth and presence of waterborne pathogens.

Keywords: coliform; drinking water; *Escherichia coli*; Polymerase Chain Reaction

Introduction

A water vending machine (WVM) is an automated self-service machine which dispenses water into the container when sufficient coins, bills or tokens are inserted (Price et al. 2006). Most freestanding floor models of WVM are located at locations such as outside grocery stores, supermarkets, or retail outlets. Access to reverse osmosis (RO), drinking water in vending machines (VMs) can improve quality of water in terms of organic, inorganic and bacteria content.

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RO can retain 99% of bacterial cell on the membrane, leaving less than 50 cell/ml in drinking water (EPA 2011; Ladner 2009).

Although well-designed WVMs are established and provided water treatments via RO, carbon filtration and UV radiation, there are still possibilities for microbe to be transmitted to water dispensers. Coliform bacteria can colonize the carbon filters of WVM resulting in high concentration of coliform bacteria in the final vended water. Suppliers or service operators of VMs need to examine the quality and safety of the water from VMs. Sampling and analyses of the vended water for bacteriological quality should be conducted to ensure public safety. Continuous monitoring of water quality from VMs and distribution parts of the VMs are essential to meet the quality requirements of ISO (WHO 2004).

However, the quality of water from VM may rapidly alter as a response to alteration in the surrounding environment of the VMs (Ali et al. 2012). Poor safety and hygiene practices when handling vending machines may transport pathogenic organisms and toxic chemicals to community which causes harm to consumers. Water contamination caused by poor sanitation and hygiene and water quality is among the top ten prevalent water-borne diseases in developing countries (Prasai et al. 2007). Inappropriate cleaning and contamination of the WVM's nozzles may result in biofilm formation and bacteria survival. According to Bloomfield et al. (2012), some heterotrophic bacteria such as *Pseudomonas aeruginosa* can adhere to the surface of WVMs such as buttons to form biofilms. Dispenser or nozzles of VMs may be contaminated with heterotrophic bacteria. Therefore, drinking water from VMs must be suitable for consumption and free from pathogenic microorganisms to ensure public safety. Hence the aim of this study was to determine the microbiological quality of water from VMs and associated contact surfaces.

Materials and Methods

Sterile Schott Duran bottles containing 2 ml of sterile 10% sodium thiosulfate were used to collect samples for microbiological analyses. Triplicate samples of 17 WVM from three locations in Kelantan (Jeli town, Tanah Merah town and an institution of higher learning in Jeli) were collected, kept in ice box containing crushed ice and transported back to the laboratory for microbiological analysis. Temperature of water samples were taken at the water vending machine sites. Each water samples (100 ml) were labelled with date and time of collection and site collection. Sterile cotton-tipped swabs were used to swab surfaces of dispense nozzles, drip trays, vending machine buttons, trap doors, coin receiving and dispensing slots. The sterile cotton-tipped swabs were dipped in sterile test tube containing 2 ml of sterile neutralizer, transferred to Whirl-Pak sampling bags, kept in ice box and transported to the laboratory. Water and swab samples collection were carried out according to APHA (1998) and Shar et al. (2008).

Sample preparation and serial dilution

A total of 1ml of water samples were added into the test tube that contains 9ml of buffered dilution water. Diluted water samples from 10^{-1} to 10^{-4} were prepared aseptically for aerobic plate counts, coliform and *E. coli* tests.

Coliform and E. coli test

Total coliform were enumerated using multiple tube fermentation technique. Complete and positive coliform test were streaked on sterile Eosin Methylene Blue (EMB) agar using spread plate method and incubated at 37˚C for 24 hours. Green metallic colonies were recognised as *Esherichia coli* and subjected to biochemical tests (methyl red, citrate and indole tests). Isolated bacteria from positive biochemical tests were selected and streaked on nutrient agar. The morphology characteristics of isolated bacteria were observed. In order to identify the bacterial strains, genomic DNA extraction, amplification and 16S rRNA sequence analysis was carried out.

Microbial culture and DNA extraction

Isolated *E. coli* colony on nutrient agar were inoculated into 10 ml trypticase soy broth (TSB) and incubated overnight at 37˚C in an orbital shaker at 150 rpm. Bacterial cultures were pelleted down, when the OD of culture reached to 0.8-1.0 at 600nm. DNA extraction was conducted to obtain the genomic DNA fragment of isolated bacteria from vending machines no. 5 and no.12 for PCR and 16S rRNA analysis. DNA extraction was conducted using G-spin Total Genomic DNA Kit (Intron, Korea).

Polymerase Chain Reaction and 16S rDNA sequence

Universal primers (Forward primer 5'-AGAGTTTGATCCTGGCTCAG-3' and reverse primer 5'-CTTGTGCGGGCCCCCGTCAATTC-3') were used for the amplification of the 16S rDNA gene fragment. PCR reaction was carried out in 50 µl reaction mixture containing: 10 ng of genomic DNA, 2.5 U of Taq polymerase, 5µl of 10X PCR amplification buffer (100 mM Tris-HCL, 500 mMKC1 pH 8.3), 200µM dNTP, 10 p moles each of the universal primers and 1.5 Mm $MgCl_2$.

Reaction was in a programmable thermal cycler (Eppendorf AG 22331 Hamburg, Germany) and the program included an initial denaturation at 94˚C for 3 minutes and then 30 cycles of denaturation at 94˚C for 30 s, annealing at 59˚C for 30 s, and extension at 72˚C for 1 min, with a final extension for 10 min at 72˚C. A 6 µl of PCR product was subjected to 1% agarose gel electrophoresis for 45 min at 80 V. Gels were stained with ethidium bromide and PCR products were visualized using a UV transilluminator and photographed. PCR products were purified using QIAquick PCR purification kit (Qiagen, Germany) and sent to First BASE Laboratories Sdn. Bhd., Malaysia for sequencing.. The sequences obtained were compared against the sequences available in the National Centre for Biotechnology Information (NCBI) database using BLAST program.

Results and Discussion

Analysis of water samples

Out of 17 samples tested, 94% of coliform bacteria were observed in 16 samples (Fig. 1). Similar results were reported by Hertin (2011), when beverage samples dispersed from 18 soda fountain machines contained 86% of coliform bacteria and exceeded the EU standard for drinking water. This may be due to insufficient cleaning and sanitation of the WVMS. Low quality of membrane filtration and lack of disinfection may contribute to bacteria re-growth after water treatment. High coliform bacteria present in drinking water also indicate that the water treatment system in VMs are not being sanitized and maintained on a regular basis. This is in agreement with Tobin et al. (1981) who mentioned that lack of maintenance on carbon filter of the vending machines may further contaminate drinking water from VMs. Poor machines condition such as missing door also increase chances of contamination of water. A study conducted by Du and Knorr (2004) reported that contamination of drinking water were attributed by poor cleanliness and maintenance services provided by the VMs owners.

Nozzles, drip trays and door swabs

A total of 13 out of 17 nozzle samples (76.47 %) were positive for coliform (Fig. 1). Nozzles were also found to contain the highest coliform count compared to other contact surfaces. According to Robertson (1987) and Lakshmanan and Schaffner (2006), nozzles may be the most soiled areas of the VMs as small volume of water still remains in the nozzle after dispensing.

14 out of 17 tray swab samples (82.35 %) were positive for coliform (>2 Most Probable Number [MPN]/100ml). Drip tray from VM no. 12 recorded the highest coliform count (> 1600 MPN/100ml). On the other hand, trays from VMs no. 10, 11, 16 and 17 showed negative results for coliform (<2 MPN/100ml). The service intervals conducted by operators were shorter for VMs no. 10, 11 and 17. Good cleaning services can minimize bacteria growth on the tray of WVM. Tray contamination could also occur when dirty bottles were placed on the tray. Eleven samples out of 17 (64.70 %) door swab samples were contaminated with coliform. Door swab sample no.12 has the highest coliform bacteria (900 MPN/100ml). The major causes of door contamination may be due to human contact by consumers with poor personal hygiene (Elalfy 2007).

Coin slots and button swabs

6 out of 17 coin slot and button swabs (35.29 %) were positive for coliform. The results indicate that both the coin slots and button swab samples have low coliform counts and do not contribute

significant contamination to the VMs. The dry environment of the coin slots and buttons may have suppressed the growth of microorganisms.

Fig. 1 Coliform count (MPN/100 ml) of water and associated surfaces of vending machines

Physico-chemical analyses

Turbidity ranged between 0.22 and 3.48 Nephelometric Turbidity Unit (NTU) with a mean turbidity of 1.06 NTU. When turbidity level exceeds 1 NTU, there is high possibility that microorganisms will be present in the water due to increased protection from disinfectant. Environmental Protection Agency (EPA, 2012) drinking water standard stated that critical acceptance level for turbidity should be between 0.5 - 1.0 NTU while IBWA (2015) stated that the turbidity in drinking water shall not exceed 0.5 NTU. Based on Table 2, 56.25% water samples exceeded the EPA drinking water standard. The highest turbidity value in water sample was found in VM no. 12 with a mean turbidity value of 3.48 ± 0.23 NTU (Table 1).

Turbidity level can be used to indicate the cloudiness of water dispensed from WVMs. High turbidity value indicates lower quality of drinking water. High turbidity is associated with higher amount of organic and particles in the water. This might protect pathogenic microorganisms (which are encased in the particles) against disinfection in WVMs (Rim et al. 2009). High turbidity value in drinking water also may be due to the presence of dust and biofilm in nozzles of WVM (Chaidez et al. 2010).

According to Ali et al. (2012), high turbidity level in drinking water may lead to illnesses such as diarrhoea and vomiting. Turbidity is a quality control parameter and can be used as an alert for operators in order to ensure effectiveness of water treatments. High total aerobic count is also associated with higher levels of turbidity which may have potential to cause illness. This can be shown in water sample no. 12 which has high turbidity and the highest concentration of coliform bacteria (>1600 MPN/100ml).

WHO and EPA recommended the pH value for drinking water should ranged from 6.5 to 8.50 (Ali et al. 2012). In this study, the water samples ranged between pH 6.23 and 8.75. This indicates 11.76% water samples exceeded the limit of acceptance of WHO and EPA. Water sample from VM no. 12 exceeded the limit of recommendation with a pH value of 8.75. pH of water outside the recommended range will have undesirable effects in terms of taste and odour (Mako et al. 2014). Poor management of membrane filtration may alter the pH of the water dispensed from WVMs.

Table 1 Physico-chemical results of water from vending machines (n=17)

Identification of bacterial strains

In total, 6 isolates were selected from positive EMB plates and biochemical tests (methyl red test, citrate test and indole test) and subcultured on nutrient agar to obtain pure culture. Basic identification of pure culture from NA was conducted to analyze the basic morphology of bacteria such as shape, nature of axis and staining colour. The morphological characteristics of isolated bacteria was summarised in Table 2. *E. coli* could be identified as circular, raised, with entire margin, opaque, small and non-endospores forming rod (State et al. 2008). Based on the morphological characteristics and reddish pink colour (Gram negative) from the Gram staining procedure, isolates from VM no. 5 and 12 were selected for 16S rRNA analysis.

Table 2 Morphological characteristics of isolated bacterial colony

Polymerase chain reaction

Genomic DNA of E2 and E6 were used in polymerase chain reaction (PCR) in thermal cycler. The purpose of PCR is to amplify the targeted region in *E. coli* from water sample and maximize selectivity for *E. coli* (Pupo et al. 1997; Sabat et al. 2000). 1 Kb ladder (Vivantis) was used to estimate molecular weight of PCR product. Based on Fig. 2 , a single and clear band of 1500 bp of 16S rRNA fragment was observed in lane 2 and 6 on agarose gel under UV light. This indicates that fragment of genomic DNA of *E. coli* was successfully amplified by the used primers 27F and 1492R which were properly bound to specific sites of the DNA template during primer annealing (Ramadan et al. 2015). The findings are similar to a study reported by Momba (2012), where all amplified PCR products from groundwater samples containing pathogenic microorganisms appear as single band of 1500bp under UV light. Sterilized nucleus free water was used in negative control instead of DNA products.

Fig. 2 PCR product after gel electrophoresis on 1.0% agarose gel

PCR purification

PCR products were purified with the QIAquick spin column to remove residual reagents used in the thermal cycler. Before sending purified product to First Base Laboratories Sdn. Bhd., Malaysia for DNA sequencing, agarose gel electrophoresis was used to confirm the presence of band inside the PCR products. The products were further subjected to DNA sequencing with the origin primers 27F and 1492R to identify strains of *E. coli*.

After receiving the DNA sequencing result from First Base Laboratories PLC, two isolates were aligned by using BLAST analysis and the identified *E. coli* strains are shown in BLAST is used to analyze the alignment by matching up each position of 16S rRNA gene sequences to each position of the sequences in the database. The percentage of similarity of isolated sample was compared with the geneBank sequence. The 16S rRNA gene from VMs no. 5 and no. 12 have been identified as *Escherichia coli* strain U 5/41 and *Escherichia coli* O157:H7 str. EDL933 with similarity value of 100% respectively.

According to Public Health England (n.d.), *Escherichia coli* U 5/41 is classified as hazard group 2 which is likely to cause human diseases. Presence of *Escherichia coli* O157:H7 str. EDL933 in water samples could be linked with biofilm formation. Biofilm formation is one of the sources that contribute to diseases in relation with public health (Beloin et al. 2008; Parsek and Singh 2003). Meanwhile, *Escherichia coli* O157:H7 is the major cause of haemorrhagic colitis and haemolytic uremic syndrome (HUS) (Andreoli et al. 2002). *Escherichia coli* O157:H7 can lead to outbreak of gastrointestinal diseases including bloody diarrhoea, kidney failure, abdominal cramps even severe hemorrhagic colitis (Peacock et al. 2001).

Conclusion

In this study, 16s rRNA sequencing identified two bacterial strains isolated from drinking water from VMs of Kelantan, Malaysia as *Escherichia coli* U 5/41 and *Escherichia coli* O157:H7 str. EDL933. The presence of pathogenic *E. coli* in drinking water poses potential threat to humans consuming the water. Regular maintenance, cleaning and sanitation of WVMs should be carried out and consumers should be educated about good personal hygiene practices to prevent cross contamination (i.e. dirty water containers in contact with drip trays, dirty hands in contact with buttons).

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Table 1 Physico-chemical results of water from vending machines (n=17)

Vending Machines	Location	Mean Turbidity (NTU)	Mean Temperature (°C)	Mean pH
1	Institute of higher learning	0.65±0.04	30.5±0.00	6.94±0.04
2		0.22±0.01	29.0±0.00	6.94±0.06
3		0.45±0.00	30.0±0.00	6.73±0.04
4		0.36±0.01	31.0±0.00	6.23±0.03
5		1.03±0.29	27.0±0.00	8.21±0.02
6		1.02±0.20	29.0±0.00	6.81±0.02
7	Jeli	0.31±0.03	27.0±0.00	6.89±0.01
8		0.40±0.02	28.0±0.00	7.17±0.03
9		1.53±0.37	29.0±0.00	6.84±0.02
10		1.67±0.26	32.0±0.00	6.84±0.01
11	Tanah Merah	1.92±0.18	31.0±0.00	7.33±0.03
12		3.48±0.23	27.9±0.00[a]	8.75±0.01
13		1.74±0.32	28.0±0.00[b]	6.90±0.01
14	Jeli	0.76±0.21	30.0±0.00[d]	6.84±0.02
15		1.14±0.05	32.0±0.00[f]	6.79±0.03
16	Tanah Merah	0.36±0.08	31.0±0.00[e]	7.31±0.01
17		1.06±0.24	31.0±0.00[e]	7.35±0.00
Average		1.06±0.83	29.6±0.00	7.11±2.55

Different superscript letters (a-e) in the same column indicate significant difference ($p<0.05$)

374 **Table 2** Morphological characteristics of isolated bacterial colony
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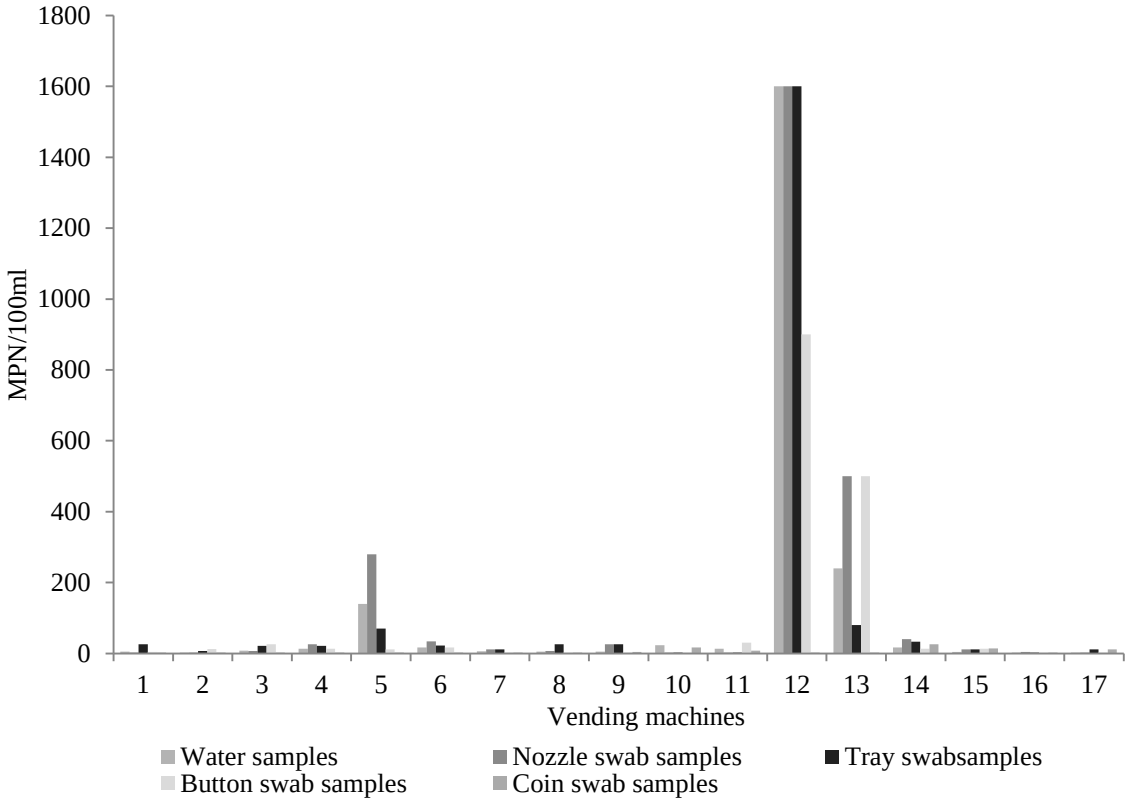
Vending machines	Shape	Size	Colony Margin	Colony Elevation	Appearance	Optical property	Texture	Pigmentation	Gram staining
5	Circular	Small	Entire	Raised	Shiny	Translucent	Smooth	No	Reddish pink
6	Circular	Small	Entire	Convex	Shiny	Translucent	Smooth	No	Purple
8	Circular	Small	Entire	Raised	Shiny	Translucent	Smooth	No	Purple
12	Circular	Small	Entire	Raised	Shiny	Translucent	Smooth	No	Reddish pink
13	Circular	Small	Entire	Raised	Shiny	Translucent	Smooth	No	Purple
16	Circular	Small	Entire	Raised	Shiny	Translucent	Smooth	No	Purple


1800
1600
1400
1200
1000
800
600
400
200
0
MPN/100ml
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
Vending machines
Water samples
Nozzle swab samples
Tray swabsamples
Button swab samples
Coin swab samples

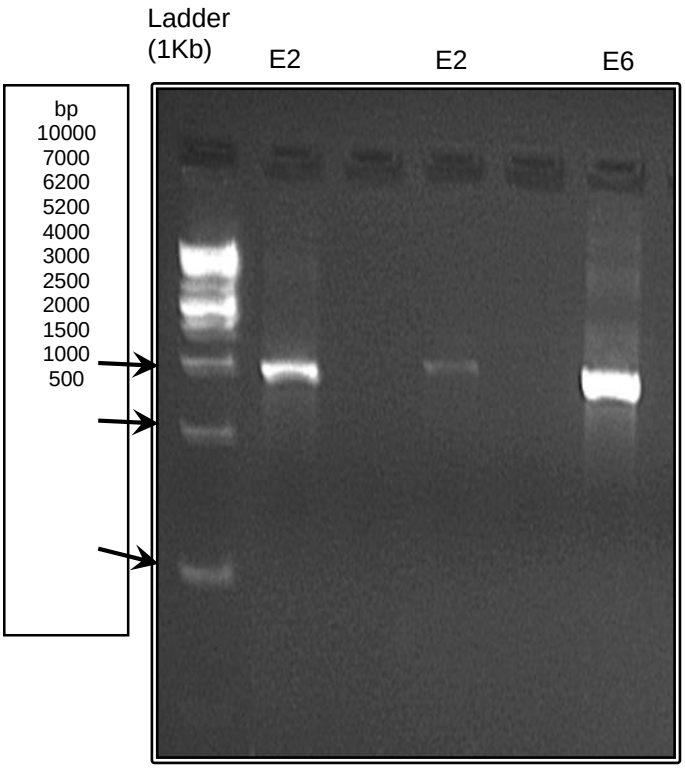


Fig. 2. Agarose gel (1%) showing amplified PCR products of 16S rDNA. Lane 1: 1Kb ladder, lanes 2 and 4: PCR product from vending machine no. 5, lane 6: PCR product from vending machine no. 12.